A Synthetic Vesicle-to-Vesicle Communication System.

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ABSTRACT: A molecular signal displayed on the external surface of one population of vesicles was used to trigger a catalytic process on the inside of a second population of vesicles. The key recognition event is the transfer of a protein (NeutrAvidin) bound to vesicles displaying desthiobiotin to vesicles displaying biotin. The desthiobiotin•protein complex was used to anchor a synthetic transducer in the outer leaflet of the vesicles, and when the protein was displaced, the transducer translocated across the bilayer to expose a catalytic head group to the internal vesicle solution. As a result, an ester substrate encapsulated on the inside of this second population of vesicles was hydrolysed to give a fluorescence output signal. The protein has four binding sites, which leads to multivalent interactions with membrane-anchored ligands and very high binding affinities. Thus biotin, which has a dissociation constant three orders of magnitude higher than desthiobiotin, did not displace the protein from the membrane-anchored transducer, and membrane-anchored biotin displayed on the surface of a second population of vesicles was required to generate an effective input signal.

INTRODUCTION

Cell membranes coordinate a large variety of biological processes by selectively recognizing and responding to different external stimuli, and membrane-spanning proteins play a vital role in these signaling pathways. Direct mass transfer by carrier proteins or by channels allows exchange of molecules between the inside and outside of the cell.[1] For signaling pathways that do not involve direct mass transfer, an external signal induces dimerization or conformational changes in membrane-spanning proteins that result in a cascade of reactions on the inner side of the membrane.[2,3] Although numerous examples of synthetic membrane channels and transporters have been reported,[4-10] signal transduction without mass transfer is considerably more challenging.[11-16]

We have recently reported a novel transmembrane signaling mechanism, which operates by controlled translocation of a synthetic transducer across a vesicle lipid bilayer (Figure 1).[17,18] The external recognition head group of the transducer becomes membrane permeable in response to an external chemical stimulus, which leads to membrane translocation, exposing a catalytic head group to the interior of the vesicle. Catalytic hydrolysis of an internal substrate generates an amplified output signal, which can also be used to trigger release of the vesicle contents.[19] The choice of recognition head group can be used to make this system respond to different external stimuli, such pH[17] or metal ions.[18] Here, we extend the scope of the signal transduction system by using ligand-protein binding as the input signal. We show that multivalent interactions at lipid bilayer interfaces lead to dramatic changes in protein-ligand binding affinities and exploit this phenomenon to achieve signal transduction processes between two different populations of vesicles.

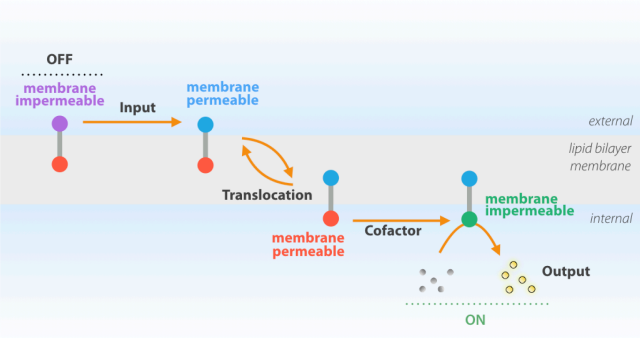


Figure 1. Signal transduction via membrane translocation. The transducer has two switchable head groups. In the OFF state, the recognition head group is membrane impermeable and sits in the external aqueous phase (purple), and the internal pro-catalyst head group sits in the membrane (red). An external signal switches the recognition head group to membrane permeable (blue), allowing translocation of the transducer across the bilayer. Binding of a charged cofactor to the catalytic head group (green) generates the ON state, where the catalyst turns over an encapsulated substrate (grey) to generate an amplified output signal (yellow).

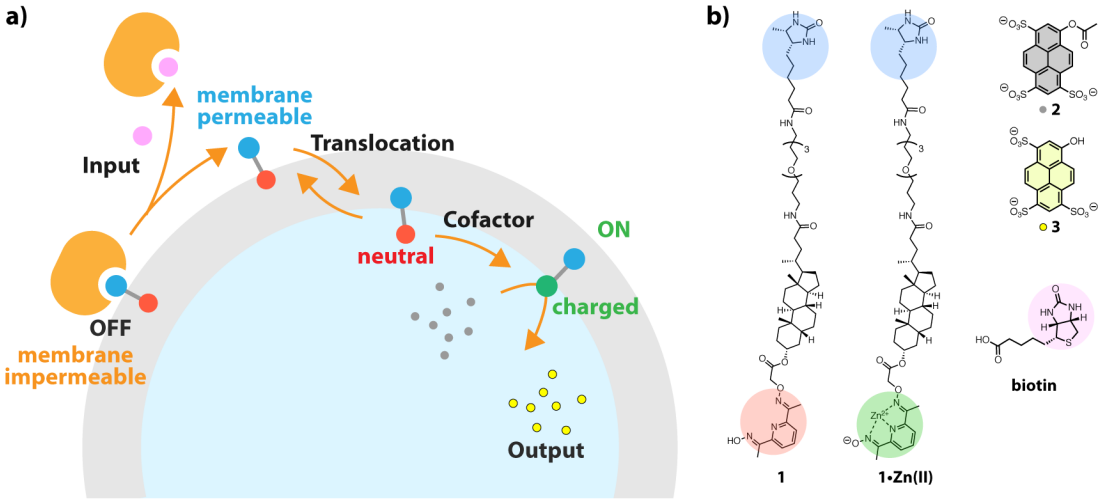


Figure 2. a) An artificial signal transduction system that responds to protein-ligand binding. In the initial OFF state, the protein-transducer complex is anchored in the outer leaflet of the vesicle bilayer and the catalytic head group (red) is buried in the membrane. The input is a competing ligand (pink) that binds the protein (orange), displacing the transducer head group (blue) and allowing it to enter the membrane. Translocation followed by binding of a charged cofactor (Zn^{2+}) from the internal aqueous solution of the vesicle activates the catalytic head group (green), which hydrolyses an internal substrate (grey) to generate an amplified output signal (yellow). b) Molecular structure of transducer **1**, the corresponding zinc complex, substrate **2**, fluorescent hydrolysis product **3**, and biotin.

APPROACH

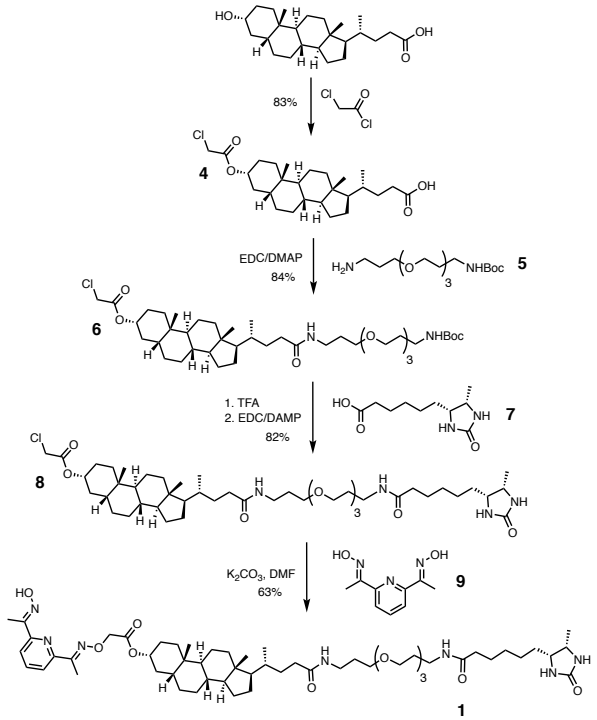
The avidin-biotin system is a well-exploited and reliable biotechnology tool used in a broad range of applications such as biochemical assays,[20] diagnosis,[21] and affinity purification.[22] Avidins are water soluble proteins that have an extremely high affinity for biotin (K_d ~ 0.01 pM) and a somewhat lower affinity for desthiobiotin (K_d ~ 10 pM).[23] The desthiobiotin-avidin-biotin system is ideally suited to construction of a transducer with a recognition head group that responds to biotin as a molecular input signal. The approach is illustrated in Figure 2a. Desthiobiotin is used as the recognition head group on transducer **1**, and assembly of vesicles in the presence of avidin should lead to the OFF state, because the desthiobiotin-avidin complex is not membrane permeable. Addition of the higher affinity ligand, biotin (pink), to the external solution should displace the protein from the vesicles, allowing the relatively non-polar desthiobiotin to enter the membrane. Translocation of the transducer followed by binding of zinc ion cofactors to the internal catalytic head group will initiate hydrolysis of ester substrate **2** inside the vesicles. The output signal in the ON state is the fluorescent emission from the hydrolysis product **3**.

The molecular structure of transducer **1** is shown in Figure 2b. The hydrophobic steroid core provides the membrane anchor, and the pyridine oxime moiety is the pro-catalyst head group that will be activated by coordination to zinc ions. The desthiobiotin recognition head group is attached via a PEG linker to make sure it can reach the avidin binding site without steric clashes between the protein and the membrane (see Supporting Information Fig. S1). We used NeutrAvidin as the protein component of this system, because it has a low isoelectric point (pI = 6.3), which minimizes protein aggregation at neutral pH.

RESULTS AND DISCUSSION

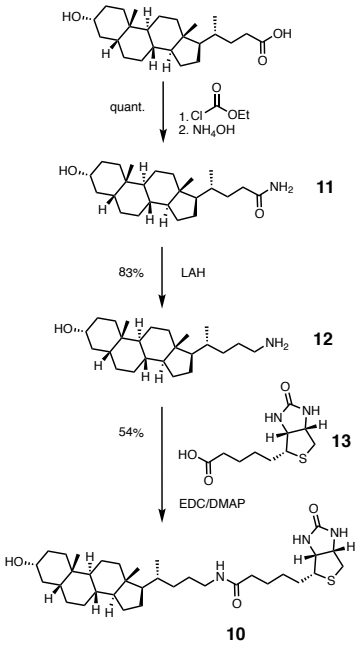
Synthesis

Synthesis of ester substrate **2** was described previously.[17] Transducer **1** was synthesized from lithocholic acid in four steps as shown in Scheme 1. Coupling of the alcohol with chloroacetyl chloride, followed by EDC coupling of the carboxylic acid with *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine **5** gave intermediate **6**. Deprotection of the amine followed by EDC coupling with desthiobiotin **7** gave **8**. Functionalization with pyridine dioxime **9** ligand gave transducer **1**.



Scheme 1. Synthesis of Desthiobiotin Transducer 1.

A membrane-anchored biotin derivative was required for the signaling experiments described below. Compound **10** was therefore synthesized from lithocholic acid using the route shown in Scheme 2. The carboxylic acid group of lithocholic acid was converted to the corresponding amine in two steps, and then EDC coupling was used to attach biotin.



Scheme 2. Synthesis of Biotin Derivative 10.

Characterization of the Input Signal

In order to confirm that NeutrAvidin binds transducer **1**, a 4'-hydroxyazobenzene-2-carboxylic acid (HABA) binding assay was first conducted in solution. HABA has a UV absorption maximum at 348 nm, which moves to 500 nm when it binds to NeutrAvidin.[24] Figure 3 shows the result of titrating a solution of transducer **1** in DMSO into a phosphate buffer solution of the HABA-NeutrAvidin complex at pH 7. The absorption at 500 nm decreased linearly with the amount of transducer until 4 equivalents had been added. Addition of just DMSO to the same solution had no effect on the absorption at 500 nm. This result indicates that, as expected, NeutrAvidin binds four equivalents of transducer **1** with a high affinity. Note that NeutrAvidin has four identical biotin binding sites, which will become important for understanding the signaling experiments below.

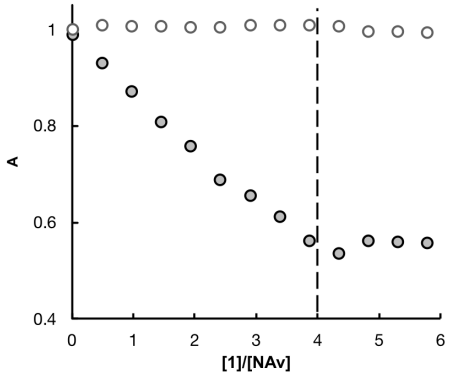


Figure 3. UV/Vis absorption titration of transducer **1** in DMSO solution (filled circles) or just DMSO (open circles) into a mixture of 0.3 mM HABA and 8 μM NeutrAvidin in 100 mM phosphate buffer with 150 mM NaCl at pH 7. The UV/Vis absorption at 500 nm is plotted as a function of the ratio of transducer [**1**] to protein [NAv].

To confirm that NeutrAvidin also binds transducer **1** when it is embedded in a vesicle membrane, we used a Förster resonance energy transfer (FRET) assay. A NeutrAvidin-TexasRed conjugate is commercially available, and nitrobenzoxadiazole (NBD) is a complementary dye suitable for FRET experiments. We prepared one set of phosphatidylcholine vesicles loaded with transducer **1** and a C6-Ceramide derivative of NBD and another set of vesicles loaded with only the dye. The NeutrAvidin-TexasRed conjugate was added to both sets of vesicles, and the results are shown in Figure 4. For the vesicles that did not contain transducer **1**, no change in the NBD fluorescence was observed, which indicates that the protein does not bind to the vesicles (Figure 4a). In contrast, when transducer **1** was also present in the vesicles, addition of NeutrAvidin-TexasRed resulted in quenching of the NBD fluorescence. The transducer-dependent quenching indicates that the protein does indeed bind to the membrane-anchored transducer, and that this interaction brings to the two dyes into sufficiently close proximity for FRET (Figure 4b). These results suggest that the NeutrAvidin•**1** complex should provide a usable OFF state for signal transduction experiments.

NeutrAvidin makes a 1:4 complex with **1**, so there are four membrane-anchors exposed on the surface of complex, and there is potential for the complex to cross-link vesicles. Careful control of stoichiometry was therefore required. For example, for the experiment shown in Figure 4, an excess of protein was used, and no vesicle aggregation was observed.

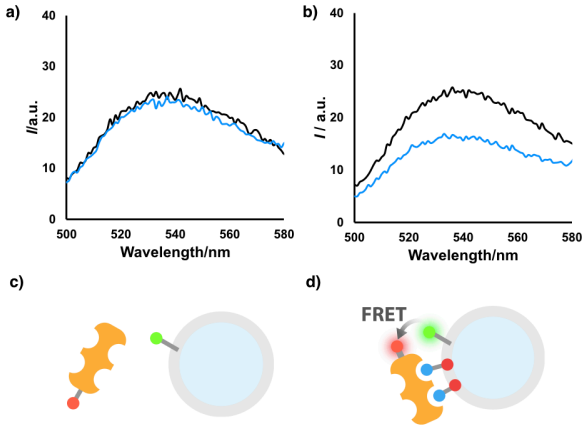


Figure 4. Fluorescence spectra (excitation at 330 nm, emission at 540 nm) of 0.01 mM 200 nm POPC vesicles with 2.5 mol% loading of NBD C6-Ceramide containing 25 mM HEPES buffer 150 mM NaCl at pH 7: a) without transducer **1**, and b) with 5 mol% loading of transducer **1**. Black: initial emission spectra, blue: spectra after addition of 0.5 μM NeutrAvidin-TexasRed (1 eq. relative to **1**). Schematic illustrations are shown in c) and d), respectively.

The same FRET assay was used to test the effect of biotin on the interaction between NeutrAvidin and transducer **1.** Vesicles loaded with transducer **1** and NBD C6-Ceramide were prepared, and the NeutrAvidin-TexasRed conjugate was added. The fluorescence quenching described above was observed, which confirms that the protein binds to the membrane-anchored transducer on the surface of the vesicles. Then biotin was added, but no change in fluorescence was observed (Figure 5a). If biotin were to dissociate NeutrAvidin from the vesicles, then the FRET between TexasRed and NBD would be abolished, which suggests that membrane-anchored desthiobiotin outcompetes biotin for protein binding. In solution, biotin binds NeutrAvidin with a 1,000-fold higher affinity than desthiobiotin. A possible reason for the change in relative affinity in the case of membrane-anchored ligands is that NeutrAvidin has four binding sites and vesicles present multiple ligands on their surface, so

multivalent interactions are possible. The protein has two binding sites on one face and two on the opposite face, so we suggest that two desthiobiotin moieties bind cooperatively to two protein binding sites at the vesicle surface, leading to an enhanced binding affinity for membrane-anchored desthiobiotin relative to solution-phase biotin (Figure 5c).[25,26]

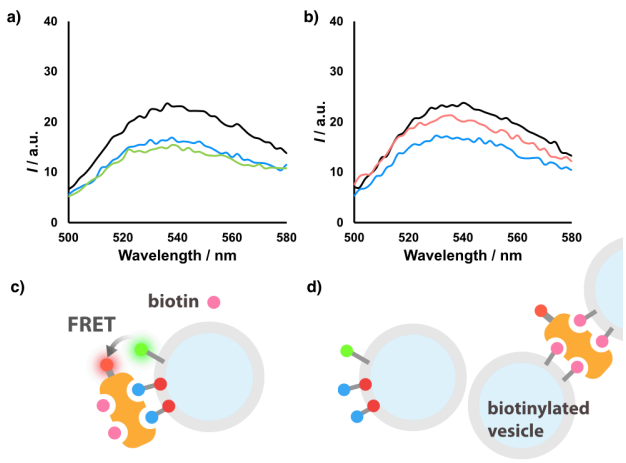


Figure 5. Fluorescence spectra (excitation at 330 nm, emission at 540 nm) of 0.01 mM 200 nm POPC vesicles with 2.5 mol% NBD C6-Ceramide and 5 mol% transducer **1** containing 25 mM HEPES buffer 150 mM NaCl at pH 7: a) initial spectrum (black), spectrum after addition of 0.5 μM NeutrAvidin-TexasRed (1 eq. relative to **1**) (blue), and spectrum after subsequent addition of 2 μM biotin (4 eq. relative to NeutraAvidin-TexasRed) (green), and b) initial spectrum (black), spectrum after addition of 0.5 μM NeutrAvidin-TexasRed (1 eq. relative to **1**) (blue), and spectrum after subsequent addition of vesicles with 5 mol% biotin derivative **10** to reach a bulk concentration of 2 μM **10** (4 eq. relative to NeutraAvidin-TexasRed) (red). Schematic illustrations are shown in c) and d), respectively.

If anchoring desthiobiotin in a membrane increases the apparent binding affinity for NeutrAvidin, the same should be true of biotin. To test this hypothesis, we used vesicles loaded with membrane-anchored biotin as the input signal to displace NeutrAvidin from the transducer. The FRET experiment described above was repeated, but instead of adding biotin to displace the NeutrAvidin from the membrane-anchored transducer, vesicles loaded with 5 mol% of biotin derivative **10** were added. The results are shown in Figure 5b. In this case, the fluorescence of the NBD present in the transducer-loaded vesicles was restored. This result shows that vesicles containing biotin derivative **10** remove NeutrAvidin from vesicles containing transducer **1**. In other words, multivalent biotin vesicles outcompete multivalent desthiobiotin vesicles, as expected from the solution-phase binding affinities. The fact that **10** binds NeutrAvidin without the PEG linker used in **1** suggests that a linker is not required for protein binding at the membrane interface. However, Figure 5b shows that the fluorescence is not fully restored by the biotin vesicles, which could be due to a relatively small difference in the protein binding affinities of the two vesicle-anchored ligands.

In these experiments, the protein was present in excess relative to **1** to avoid cross-linking of the vesicles, so the other protein binding sites were initially empty. Addition of the biotin vesicles could cause cross-linking due to multivalent interactions at both vesicle membrane interfaces, but an excess of biotin was used relative to transducer **1**, and no aggregation was observed on the timescale of the measurements. The reorganization of multivalent interactions that would lead to vesicle aggregation appears to be relatively slow. However, when solutions containing both sets of vesicles were stored overnight, visible flocculation was observed indicating that aggregation of this system does take place on longer timescales.

Transmembrane Signaling Experiments

Before carrying out signaling experiments, we tested the catalytic activity of the transducer in the ON and the OFF states. Vesicles were prepared containing 250 μM ester **2** and 250 μM zinc chloride in HEPES buffer at pH 7. When transducer **1** was added to this solution, it inserted into the membrane and initiated catalysis of ester hydrolysis inside the vesicles (green data in Figure 6a). This system corresponds to the ON state of the signal transduction system and shows that the desthiobiotin head group does not interfere with catalysis. When the NeutrAvidin•**1** complex was added to the vesicle solution, no increase in the background rate of hydrolysis of substrate **2** inside the vesicles was observed (black data in Figure 6a). This system corresponds to the OFF state of the signal transduction system and shows that protein binding holds the catalytic head group inside the membrane, preventing translocation. Vesicle cross-linking in this OFF state was avoided by adding an excess of the NeutrAvidin•**1** complex to ensure that the surfaces of the vesicles were saturated.

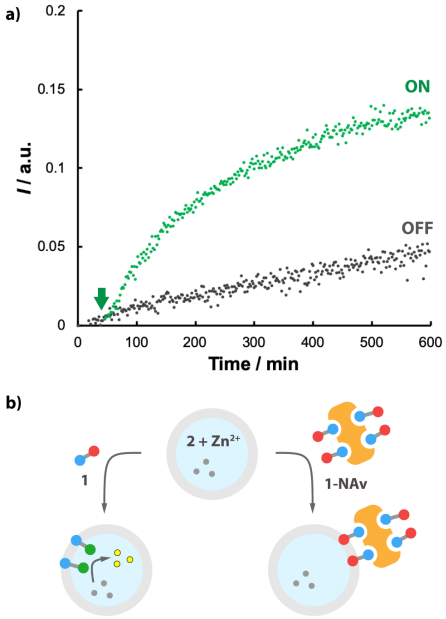


Figure 6. a) Time dependence of the normalized fluorescence emission intensity at 510 nm (exciting at 415 nm). The ON state (green) was obtained by adding transducer **1** in DMSO to vesicles to reach 10 mol% loading in lipids and a bulk concentration of 10 μM **1** (addition at time point indicated by the arrow). The OFF state (black) was obtained by adding the NeutrAvidin•**1** complex (17 μM protein, 67 μM **1**, i.e. 0.25 eq. of protein eq. relative to **1**). All experiments were conducted in 0.1 mM 200 nm DOPC/DOPE vesicles containing 250 μM **2**, 250 μM zinc chloride, and 25 mM HEPES buffer 150 mM NaCl at pH 7. b) Schematic illustration.

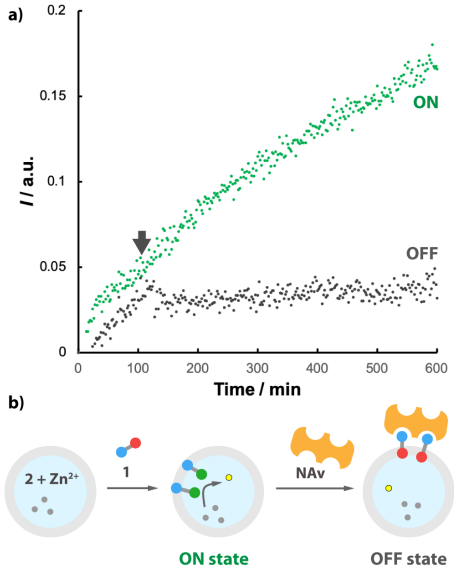


Figure 7. a) Time dependence of the normalized fluorescence emission intensity at 510 nm (exciting at 415 nm). The ON state (green) was obtained by adding transducer **1** in DMSO to vesicles to reach 10 mol% loading in lipids and a bulk concentration of 1 μM **1**. After 100 minutes, 2 μM NeutrAvidin was added (2 eq. relative to **1**) to generate the OFF state (black, addition indicated by the arrow). All experiments were conducted in 0.01 mM 200 nm DOPC/DOPE vesicles containing 250 μM **2**, 250 μM zinc chloride, and 25 mM HEPES buffer 150 mM NaCl at pH 7. b) Schematic illustration.

Having demonstrated that the ON and OFF states function as anticipated, we then attempted to switch between these two states by using external signals to initiate signal transduction. The ON state was assembled as described above, and NeutrAvidin was added to the vesicle suspension after two hours. Figure 7a shows that addition of the protein efficiently switches the system from the ON to the OFF state. In this case, two equivalents of protein relative to transducer **1** were used to avoid vesicle cross-linking, and this stoichiometry is more effective at suppressing the reaction than the 0.25 equivalents of NeutrAvidin used for the experiments shown in Figure 6a.

To demonstrate switching from the OFF to the ON state, the OFF state was assembled as described above by adding the NeutrAvidin•**1** complex to vesicles containing substrate **2** and zinc ions. Figure 8a shows the effect of adding biotin to this system. Biotin slightly reduced the background rate of substrate hydrolysis (red data), but did not generate an ON state, as expected from the FRET experiments described above. Even a saturated solution of 1 mM biotin produced no signs of an ON signal (purple data). However, addition of vesicles loaded with 10 mol% of biotin derivative **10** lead to a rapid increase in fluorescence emission characteristic of the ON state (green data in Figure 8a). When the loading of **10** in the biotin vesicles was lowered to 5 mol%, an ON state was also obtained, albeit with lower catalyst activity (blue data). This result suggests that the affinity of the biotin vesicles for NeutrAvidin can be controlled with vesicle loading by changing the effective molarity for the cooperative binding interactions at the membrane interface. The timescale for the signaling experiments is significantly longer than for the FRET experiments, so flocculation was sometimes observed at the end of these experiments, presumably due to vesicle cross-linking. The experiments shown in Figure 8 indicate that signaling molecules displayed on the surface of one set of vesicles are able to initiate catalytic substrate turnover inside a second set of different vesicles. This process is reminiscent of the kind of complex cell-to-cell signaling processes found in biological organisms.

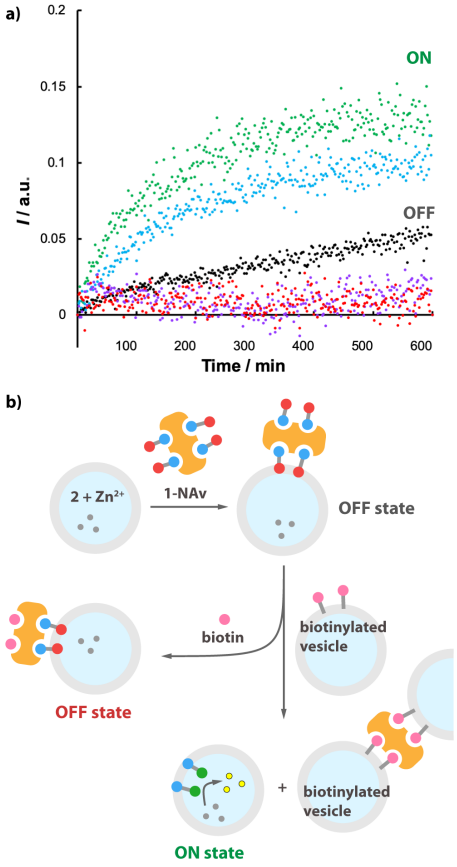


Figure 8. a) Time dependence of the normalized fluorescence emission intensity at 510 nm (exciting at 415 nm). The OFF state (black) was obtained by adding NeutrAvidin•**1** complex (17 μM protein, 67 μM **1**, i.e. 0.25 eq. of protein eq. relative to **1**) to 0.1 mM DOPC/DOPE vesicles containing 250 μM **2**, 250 μM zinc chloride, and 25 mM HEPES buffer 150 mM NaCl at pH 7. Addition to the OFF vesicle solution of either 67 μM biotin (4 eq. relative to NeutrAvidin, red) or 1 mM biotin (60 eq. relative to NeutrAvidin, purple) biotin both gave OFF states. Addition to the OFF vesicle solution of different amounts of 200 nm DOPC/DOPE vesicles loaded with 10 mol% of biotin derivative **10** (green) or 5 mol% of biotin derivative **10** (blue) to reach bulk concentrations of 67 μM **10** (4 eq. relative to NeutrAvidin) both gave ON states. b) Schematic illustration.

CONCLUSIONS

The system described here constitutes the first example of a synthetic construct where cooperative interactions at membrane interfaces have been used to trigger transmembrane signal transduction. A molecular signal (biotin) displayed on the external surface of one population of vesicles was used to trigger a catalytic process on the inside of a second population of vesicles. The key recognition event is the exchange of proteins (NeutrAvidin) bound to vesicles displaying desthiobiotin to vesicles displaying biotin. The desthiobiotin•NeutrAvidin complex was used to anchor a synthetic transducer in the outer leaflet of the vesicles, and when the protein was displaced, the transducer translocated across the bilayer to expose a catalytic head group to the internal vesicle solution. As a result, an ester substrate encapsulated on the inside of this second population of vesicles was hydrolysed to give a fluorescent product, which

constitutes an amplified output signal. NeutrAvidin has four ligand binding sites, and multivalent interactions with the membrane-anchored ligands leads to very high binding affinities. Thus biotin, which has a dissociation constant three orders of magnitude higher than desthiobiotin, did not displace NeutrAvidin from the membrane-anchored transducer, and membrane-anchored biotin was required to generate the input signal. These findings extend the scope of artificial signal transduction from purely synthetic assemblies into more sophisticated systems, which opens up the potential for future development of intelligent responsive vesicles in bionanotechnology.

ASSOCIATED CONTENT

Supporting Information

Materials and methods, experimental details, synthetic procedures and compound characterization are available in the Supplementary Materials. This material is available free of charge via the Internet at http://pubs.acs.org.

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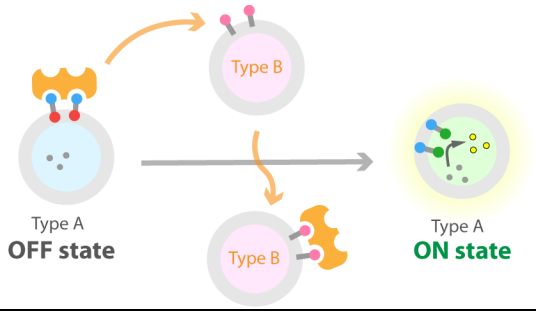
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